Differential Expression on a Daily Basis of Plastid Sigma Factor Genes from the Moss Physcomitrella patens. Regulatory Interactions among PpSig5, the Circadian Clock, and Blue Light Signaling Mediated by Cryptochromes

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The nuclear-encoded plastid sigma factors are supposed to be a regulatory subunit of the multisubunit bacteria-type plastid RNA polymerase. We studied here whether or not three genes, PpSig1, PpSig2, and PpSig5 encoding plastid sigma factors, are controlled by the circadian clock and/or by blue light signaling in the moss Physcomitrella patens. Among the three PpSig genes, only PpSig5 was clearly controlled by the circadian clock. In contrast to the differential regulation on a daily timescale, a pulse of blue light induced the expression of all the three PpSig genes. This induction was significantly reduced in a knockout mutant that lacked the blue light photoreceptor cryptochromes PpCRY1a and PpCRY1b, indicating that PpCRY1a and/or PpCRY1b mediate the blue light signal that induces the expression of the PpSig genes. In a daily cycle of 12-h blue light/12-h dark, the timing of peak expression of PpSig5 and a chloroplast gene psbD, encoding the D2 subunit of photosystem II, advanced in the cryptochrome mutant relative to those in the wild type, suggesting the presence of regulatory interactions among the expression of PpSig5 and psbD, the circadian clock, and the blue light signaling mediated by the cryptochrome(s).

Plastids contain their own genome owing to their free-living bacterial ancestry. At least two distinct RNA polymerases transcribe plastid genes in higher plants: one is the nuclear-encoded plastid RNA polymerase and the other is the primarily plastid-encoded plastid RNA polymerase (PEP; Gray and Lang, 1998). Nuclear-encoded plastid RNA polymerase is a single-subunit bacteriophage-type enzyme and is thought to generally transcribe housekeeping genes including the PEP core enzyme genes (Stern et al., 1997; Weihe and Borner, 1999). On the other hand, PEP is a multisubunit bacteria-type enzyme and consists of four plastid-encoded subunits of the core enzyme, α, β, β’, β”, and a nuclear-encoded plastid sigma factor (Allison, 2000). The plastid sigma factor is supposed to define binding specificity to target promoter sequences, based on the functions of eubacterial counterpart sigma-70 family proteins (Allison, 2000). The bacterial genome encodes multiple sigma factors with different promoter specificities, enabling transcriptional activation of different sets of genes in response to various endogenous and environmental signals (Gruber and Gross, 2003). Recent studies have suggested this picture to be also applicable to the plastid sigma factors. The Arabidopsis (Arabidopsis thaliana) genome contains six distinct sigma genes from AtSig1 to AtSig6, each of which, except for AtSig4, orthologous genes have been found from various higher plant species (Fujiwara et al., 2000; Tsunoyama et al., 2004). Therefore, the plant sigma factor family is subdivided into structurally, and presumably functionally, different groups beyond species. Recently, AtSig2 and AtSig5 have been shown to define respective sets of target chloroplast genes albeit with some overlaps; moreover, AtSig5 mediates specific stress stimuli such as high light and low temperature to the target genes (Kanamaru et al., 2001; Hanaoka et al., 2003; Privat et al., 2003; Nagashima et al., 2004; Tsunoyama et al., 2004). On the other hand, the functions and target genes of sigma factors other than AtSig2 and AtSig5 remain unknown.

The circadian clock is an autonomous oscillator with an endogenous period of approximately 24 h, and it controls a wide variety of processes from gene expression to leaf movements for the adaptation to the environment that changes on a daily timescale (Young...
and Kay, 2001). In Arabidopsis, “clock genes” encoding the candidate components of the core clock machinery have been isolated (Hayama and Coupland, 2003). Currently, functions and interactions of clock genes and their encoded proteins (clock proteins) are being studied intensively to unravel the molecular mechanisms of the clock (Hayama and Coupland, 2003). The clock controls many genes (called clock-controlled genes [ccgs]) on the nuclear and chloroplast genomes, resulting in circadian rhythms of the transcript levels of the ccgs even under constant conditions (Nakahira et al., 1998; Harmer et al., 2000; Schaffer et al., 2001). Candidate clock proteins CCA1 (circadian clock-associated-1) and LHY (elongated hypocotyl), myb transcription factors, seem to control the rhythmic expression of certain sets of ccgs on the nuclear genome by directly binding to its promoters (Wang et al., 1997; Alabadi et al., 2001). By contrast, it is still almost totally unknown how temporal information is transmitted from the clock to chloroplast genes. In cyanobacteria, RpoD2, one of the sigma-70 family proteins, revealed to mediate between the clock and a set of downstream genes (Tsinoremas et al., 1996).

Given that plastids arose through endosymbiosis from a photosynthetic bacterium closely related to extant cyanobacteria, the function of RpoD2 might be conserved in a plastid sigma factor(s). This idea is supported by the observations that some of the plastid sigma factor genes show circadian rhythms in transcript accumulations (Morikawa et al., 1999; Harmer et al., 2000), though its direct role on the chloroplast gene expression has not yet been examined. Nakahira et al. (1998) reported a robust circadian rhythm in the transcription from the blue light responsive promoter (BLRP) of the psbD gene, encoding the D2 protein of PSII. Since an unusual −35 sequence was critical for the rhythmic expression of psbD gene, they argued that a sigma factor(s), known to recognize the −35 and −10 sequences of a bacterial type promoter (Allison, 2000), might transmit temporal information from the clock to BLRP (Nakahira et al., 1998). BLRP is controlled not only by the clock, but also directly activated by blue light, as inferred from its name (Stern et al., 1997). The plastid sigma factors have been candidate proteins mediating light-inducible expression of plastid genes, because many sigma genes are light-inducible (Allison, 2000). Very recently, this was reported to be the case for BLRP; AtSIG5 protein mediates between a blue light signal from the blue light photoreceptor cryptochromes and transcription from BLRP (Nagashima et al., 2004; Tsunoyama et al., 2004). Collectively, the plastid sigma factor is expected to be key a regulator integrating different regulations such as the clock and light, and hence its study is critical for understanding the regulatory mechanisms underlying the daily expression profiles of plastid genes in daily light-dark cycles.

Physcomitrella patens is a newly established model plant to which gene targeting with exceptionally high efficiency for a plant can be easily applicable (Schaefer, 1994). In addition, P. patens is an interesting plant from an evolutionary point of view because it is classified into bryophyte, which divided from other plant lineages at a very early stage of the evolution of land plants (Heckman et al., 2001; Schaefer and Zryd, 2001). The regulatory system of plastid gene expression has drastically changed during the evolution of photosynthetic organisms (Sato, 2001). Based on these facts, we chose P. patens as the experimental plant for the functional study of plastid sigma factors (Hara et al., 2001a, 2001b). We isolated two distinct plastid sigma factor genes PpSig1 and PpSig2 encoding PpSIG1 and PpSIG2 proteins, respectively, from P. patens (Hara et al., 2001a, 2001b). PpSIG1 and PpSIG2 are closely related to the higher plant Sig1 and Sig2 groups, respectively; that is, the Sig1 and Sig2 groups were already present in the common ancestor of the bryophyte and higher plants (Hara et al., 2001a, 2001b).

Here we report a newly isolated cDNA species PpSig5 encoding another P. patens plastid sigma factor, which is classified into the most diverged Sig5 group. We examined: (1) whether the expression of the PpSig genes is controlled by a circadian clock; and (2) how the blue light signaling mediated by cryptochromes affects the daily expression patterns of the PpSig genes using a mutant strain that lacked PpCry1a and PpCry1b, encoding the blue light photoreceptor cryptochromes. The results demonstrate that the expression of the newly isolated PpSig5 shows a unique feature among the three sigma factor genes in P. patens.

RESULTS

Identification of PpSig5 Gene in P. patens

By a database (the Physcomitrella EST Programme) search using the BLAST program we found an expressed sequence tag (EST) sequence (accession no. BI894524) that was similar to plastid sigma factor sequences from various plants but was identical to neither PpSig1 nor PpSig2 sequences. We isolated the full-length cDNA by screening a cDNA library (a gift from the Physcomitrella EST Programme) using this EST sequence as a probe. The amino acid sequence deduced from the predicted coding region of the cDNA showed the highest similarity to Arabidopsis AtSIG5 sequence (identity and similarity were 37% and 58%, respectively). All the domains conserved among plant sigma factors (domains 1.2–4.2 [Fig. 1A]) were present in the novel P. patens sigma factor sequence. To examine the evolutionary relationships between this sigma factor and the plastid sigma factors reported to date, a phylogenetic tree was constructed based on the sequences of the novel sigma factor and 22 plastid sigma factors from various seed plants and P. patens. As described previously (Hara et al., 2001b), the plastid sigma factors were divided into some clusters in the tree (Fig. 1B). The novel sigma factor was clustered together with AtSIG5 and OsSIG5, indicating that it is classified into the Sig5 group of
Figure 1. (Legend appears on following page.)
the plastid sigma factor family. This result was supported by a comparison of intron positions on the coding sequences of Arabidopsis and P. patens sigmas (Fig. 1A). The intron positions of the novel sigma factor and AtSIG5 were found completely at identical positions by the alignment of amino acid sequences. Based on these results, we speculated that the novel sigma cDNA encodes the third plastid sigma factor in P. patens and named the corresponding gene and its protein product PpSig5 and PpSIG5, respectively.

Diurnal Changes in mRNA Accumulation of Three PpSig Genes and psbD gene in LD

We examined the temporal patterns of the expression of three distinct P. patens sigma factor genes (PpSig1, PpSig2, and PpSig5) under three different light regimens to study if the sigma genes are under the control of the circadian clock. First, we measured the changes of the three PpSig mRNA accumulations for three 12-h-light/12-h-dark cycles (LD) by semi-quantitative reverse transcription (RT)-PCR analyses (Fig. 2A). The PpSig1 and PpSig2 genes were expressed throughout a day, with fluctuations that suggest diurnal rhythms of mRNA levels with very low amplitude. By contrast, PpSig5 mRNA showed a very high-amplitude diurnal rhythm with peaks observed in light phases. In dark phases, the PpSig5 mRNA levels were close to the detection limit and started to increase before the onset of light. We measured the mRNA levels for 24 h at intervals of 2 h under LD (Fig. 2B). The PpSig1 mRNA levels showed slight increases at the onset of light and dark phases, whereas the PpSig2 mRNA levels showed a monophasic diurnal rhythm with a peak in the light phase and a trough in the dark phase. The PpSig5 peaked 4 to 6 h after the onset of light and showed a trough at 2 to 6 h in the dark. Then it started to increase around 4 h before the onset of light. The predawn “anticipatory” rise of PpSig5 expression, together with very high peak-to-trough ratio of the oscillation, suggests that PpSig5 is under the strong control of an endogenous circadian clock.

We also measured the changes in mRNA accumulation of a chloroplast gene psbD, which encodes the D2 protein of PSII. In wheat (Triticum aestivum), the expression of psbD is reported to be controlled by a circadian clock (Nakahira et al., 1998). The P. patens psbD showed a diurnal rhythm with peaks observed in light phases (Fig. 3, A and B). The predawn rise was also observed for psbD, suggesting that psbD is also controlled by the clock in P. patens.

Changes in mRNA Accumulation of PpSig and psbD Genes in Constant Conditions

The most reliable diagnostic feature of circadian rhythms is that they persist under constant conditions. Therefore, we measured the expression of the PpSig and psbD genes in continuous light (LL; Fig. 4), or in continuous dark (DD; Fig. 5) after entraining the clock in LD.

In LL, we could not obtain any signs of circadian control on any of the genes tested (Fig. 4). The PpSig1 and PpSig2 mRNA levels did not show significant fluctuation throughout the monitoring period. The PpSig5 and psbD mRNA levels peaked immediately after the transfer from LD to LL, corresponding to the peaks observed in LD. However, the PpSig5 mRNA levels did not show severe reduction after this peak, unlike the patterns observed in LD.

In DD, the three PpSig genes showed characteristic patterns that were distinct from each other (Fig. 5A). The PpSig1 mRNA levels rapidly decreased in the latter half of the first day in DD, and only very low levels, if any, of expression were detected after 1 d in DD. The PpSig2 mRNA levels showed a gradual decrease in DD, while its expression was still observed even 2 d after the onset of DD. The PpSig5 mRNA levels showed a clear peak in the early half of the first subjective day phase (subjective day and night in continuous conditions correspond to light and dark phases, respectively, in the preceding light-dark cycles) and a lower, but reproducible, peak in the second subjective day. These observations, together with those in LD and LL experiments, clearly indicate that three sigma genes are differentially regulated on the daily timescale. In particular, the rhythmic expression of PpSig5 in LD and DD indicates that the circadian clock controls this gene. In DD, the psbD gene also showed rapidly damping oscillation with phasing similar to
that of \( \text{PpSig5} \), though with a much lower amplitude, suggesting that \( \text{psbD} \) is also under the control of the clock (Fig. 5B).

**Expression of \( \text{PpRpoA} \) Gene**

In \( P. \text{patens} \), the nuclear gene \( \text{PpRpoA} \) encodes the \( \alpha \)-subunit of PEP (Sugiura et al., 2003). We also measured the temporal changes in the \( \text{PpRpoA} \) mRNA levels (Fig. 6). In LD (Fig. 6, A and B), the \( \text{PpRpoA} \) mRNA levels gradually increased in light and decreased in dark, resulting in a diurnal rhythm with a very low amplitude with peaks and troughs observed at the offset and onset of light, respectively. In LL, after the gradual increase in the first 12 h, the mRNA levels did not fluctuate greatly (Fig. 6C). In DD, after the decrease in the first 12 h, the mRNA levels did not fluctuate and remained relatively low over the monitoring period (Fig. 6D). These expression patterns of \( \text{PpRpoA} \) suggested that the diurnal rhythm of \( \text{PpRpoA} \) in LD resulted from direct but weak up-regulation by light, not from the endogenous regulation by the clock.
The results are consistent with those in wheat, where the expression of a sigma factor gene (TaSig1) showed a circadian rhythm while that of rpoA did not (Morikawa et al., 1999).

Effect of Blue Light Signaling Mediated by Cryptochromes on Induction of PpSig, PpRpoA, and psbD Genes

Recent studies indicated that the blue light signal mediated by cryptochromes induced the transcription from BLRP via AtSIG5 protein (Thum et al., 2001; Nagashima et al., 2004; Tsunoyama et al., 2004). We examined if the blue light signaling mediated by cryptochromes induces the PpSig, PpRpoA, and psbD genes in P. patens. First, we tested the light-inducibility of PpSig, PpRpoA, and psbD genes in wild-type cells. We previously demonstrated that white light induced the expression of PpSig1 and PpSig2 (Hara et al., 2001b). We observed here that white light induced PpSig5, PpRpoA, and psbD as well as PpSig1 and PpSig2 (Fig. 7). The induction rates of PpSig2, PpRpoA, and psbD were not as large as those of PpSig1 and PpSig5, reflecting that the former genes retained significant levels of expression even after exposure to darkness for 24 h (Fig. 5, A, B, and D). We also observed that a blue light pulse also induced the expression of all genes tested in wild-type cells (Fig. 7). Next, we tested the light-inducibility of the genes in a cryptochrome mutant. Imaizumi et al. (2002) identified from P. patens two cryptochrome genes, PpCry1a and PpCry1b, encoding PpCRY1a and PpCRY1b proteins, respectively. They demonstrated by the gene targeting experiments...
that PpCRY1a and PpCRY1b have redundant functions, being supported by the fact that there was only one amino acid difference between PpCRY1a and PpCRY1b (Imaizumi et al., 2002). Based on these observations, we used a double disruption mutant of PpCry1a and PpCry1b, where more severe phenotypes were expected than the single disruption mutant of either gene (Imaizumi et al., 2002). Imaizumi et al. (2002) developed this mutant strain by integrating the hpt (hygromycin B phosphotransferase) and nptII (neomycin phosphotransferase II) gene cassettes into the coding regions of PpCry1a and PpCry1b, respectively. In the mutant strain, the induction of the three PpSig genes and PpRpoA gene by blue light was significantly reduced relative to those in wild-type cells (Fig. 7, A–D), indicating that either or both of the cryptochromes mediated the blue light signal inducing these genes. The induction of PpSig1 and PpSig5 by blue light was only partially reduced in the mutant, indicating that a blue light photoreceptor(s) other than the two cryptochromes is involved in the induction. On the other hand, induction of the three PpSig genes and PpRpoA gene by white light was not, if at all, significantly reduced in the mutant relative to that observed in wild-type cells. This result indicated that photoreceptors sensing wavelengths other than blue light also contribute to the light induction of these genes. As for the psbD gene, we could not obtain data indicating the involvement of the cryptochrome(s) in the induction by blue light (Fig. 7E).

Figure 4. Changes in abundance of mRNA for three PpSigs and psbD under LL. P. patens cells were maintained in LD for more than 3 weeks after which cells were harvested in LL (40 μmol m⁻² s⁻¹) at indicated times for 2 d. The abundance of PpSigs mRNAs and psbD mRNAs were measured as described in legends for Figures 2 and 3, respectively. A, Changes in abundance of PpSig1, PpSig2, and PpSig5 mRNAs. White circles, PpSig1; black circles, PpSig2; white triangles, PpSig5. B, Changes in abundance of psbD mRNA. White and hatched bars indicate light intervals and subjective dark intervals, respectively. We obtained similar results in two independent experiments.
Effect of Blue Light Signaling Mediated by Cryptochromes on the Daily Expression Profiles of PpSigs, PpRpoA, and psbD Genes

To study the effect of the blue light signal via cryptochromes on the diurnal expression profiles of PpSigs, PpRpoA, and psbD, we compared the changes in the mRNA levels of these genes in a 12-h-blue light/12-h-dark cycle (BLD) between wild-type cells and the cryptochrome mutant cells. The accumulation of all genes tested was reduced in a time-dependent manner in the disruption mutant (Fig. 8). Especially, the PpSig5 mRNA levels were more severely reduced in the latter half of the blue light period (hours 8 and 12) than at other time points, resulting in the apparent phase advance of the diurnal rhythm of PpSig5. The kinetics of psbD was also altered in a way similar to that of PpSig5 in the mutant (Fig. 8, C and E), although the reduction of the average level of expression of psbD was not as large as that of PpSig5. The results indicate that the blue light signaling via cryptochrome(s) influences the daily expression profiles of tested genes.

DISCUSSION

This is the first demonstration of the presence of a Sig5 ortholog gene in a plant other than angiosperms (Tsunoyama et al., 2004). The identification of PpSig1, PpSig2 (Hara et al., 2001b), and PpSig5 (this study) genes, together with the ancient phylogenetic origin of bryophytes, indicates that the members of the plant
sigma family were diverged at very early stages in the evolution of land plants. It remains to be seen how much further the diversification of the sigma family can be traced back in the plant lineages. Interestingly, the Sig5 sequences are always found to be positioned outside all the other sigma groups in the phylogenetic trees (Fig. 1B; Allison, 2000; Fujiwara et al., 2000; Hara et al., 2001b; Tsunoyama et al., 2004), suggesting that the Sig5 group originated still earlier than any other sigma groups. This idea is supported by the fact that the distribution of intron positions of AtSig5 and PpSig5 is unique among the sigma factor genes from P. patens and Arabidopsis (Fig. 1A; Allison, 2000; Fujiwara et al., 2000; Tsunoyama et al., 2004).

This study demonstrated with a high-density time resolution that the expressions of the P. patens sigma genes are regulated differently from each other on a daily timescale, reflecting different dependency of the three genes on light and the circadian clock. In nature, environmental factors such as light and temperature, which strongly affect photosynthesis, change with a period of 1 d due to the earth's rotation. The dependency of respective photosynthesis genes on such environmental factors differ from each other, depending on each gene's function. Therefore, to achieve efficient photosynthesis under alternating day and night cycles, it is supposed to be very important to activate a particular set(s) of chloroplast genes at a specific time of day. It is very likely that differential use of plastid sigma factors on a daily basis underlies such complex regulation. The most contrasting point in the expression of three sigma genes is that only PpSig5 is under the strong control of the circadian clock. The PpSig1 and PpSig2 genes were expressed in both day and night, though with low-amplitude fluctuations (Fig. 2), suggesting that PpSIG1 and PpSIG2 proteins might regulate the genes whose protein products are required throughout a day. The Arabidopsis AtSIG2 protein is known to activate the transcription of chloroplast tRNA genes (Kanamaru et al., 2001). This observation is consistent with the result obtained in P. patens, because tRNA genes should be expressed throughout a day for enabling translation of different mRNA species with various expression profiles. In sharp contrast to other two PpSig genes, PpSig5 showed very low levels of expression in the dark phases (Fig. 2), being controlled by the clock. The Arabidopsis AtSIG5 protein, when
overexpressed, enhanced the expression of psbA, psaA, psbB, and psbD genes, indicating these photosystem genes to be possible targets of AtSIG5 (Tsunoyama et al., 2004). The P. patens psbD gene showed expression with temporal profiles similar to that of PpSig5 (Figs. 2–5), suggesting a possibility that psbD may be a target gene of Sig5 protein in P. patens as well as in Arabidopsis. It seems reasonable that a positive regulator of the photosystem genes shows a daily rhythm that ensures a sufficient level of photosystem proteins in the light. However, we do not yet have evidence that PpSIG5 regulates the expression of photosystem genes including psbD gene. To directly examine this issue, targeted disruption of PpSig5 followed by the expression analysis of chloroplast genes should be carried out.

An interesting possibility is that PpSig5 may be involved in adaptation to stress conditions. It is well known that the PSI reaction center is damaged by high light, especially when combined with other environmental stresses like low temperature (Giardi et al., 1997). Nagashima et al. (2004) postulated that AtSIG5 repairs such damaged PSI by inducing the expression of PSI genes in response to stresses such as high light and low temperature. The coincidence of strong light and low temperature, possibly damaging PSII, is expected to occur in the morning. If the function of AtSIG5 postulated by Nagashima et al. (2004) is conserved in PpSIG5, the rhythmic expression of PpSig5, peaking in the light phase with anticipatory predawn rise, is a plausible mechanism for protection against rhythmic changes of environmental stresses. We do not yet know the functions of PpSIG5. However, PpSig5 is induced by high light, whereas other PpSig genes are not (K. Ichikawa and S. Aoki, unpublished data), suggesting that Sig5 gene in P. patens as well as in angiosperm may also be involved in the stress adaptation like its counterpart in angiosperm. In P. patens, not only gene disruption but also promoter replacement is applicable (Schaefer and Zryd, 2001). By using such a technical advantage of P. patens, it will be possible to generate strains with different phasing of PpSig5 expression, with which the physiological significance of rhythmic expression of PpSig5 can be addressed.

Interestingly, neither PpSig5 nor psbD showed rhythmic expression in LL (Fig. 4), which is in contrast to higher plants, in which rhythmic expression of the sigma and psbD genes was clearly observed in LL (Nakahira et al., 1998; Morikawa et al., 1999). Likewise, we reported that the expression of other ccs genes in P. patens (Lhcb and PpCOL1 genes) was arrhythmic in LL,
Figure 8. Effects of cryptochrome mutations on diurnal expression profiles of PpSigs, psbD, and PpRpoA genes. Protonema cells of both wild-type (WT) and cry1a cry1b (ΔCry) were maintained in LD for more than 2 weeks and then transferred to a BLD. Abundances of the PpSigs, PpRpoA, and psbD mRNAs were measured in samples harvested at indicated times in BLD as described in previous figures. The top sections show the changes of the relative levels of each gene’s mRNA. Values are means ± SD from three independent experiments. Striped and black bars indicate blue light intervals (40 μmol m⁻² s⁻¹) and dark intervals, respectively. White circles, wild-type; black circles, cry1a cry1b. The bottom sections show the hybridized bands for each test gene and actin or 18S rRNA gene. Asterisks show genomic amplifications of the PpRpoA gene. A, PpSig1; B, PpSig2; C, PpSig5; D, PpRpoA; E, psbD.
whereas they showed damping oscillation in DD (Aoki et al., 2004; Shimizu et al., 2004). These observations are also in contrast to higher-plant counterpart genes, which were reported to show robust circadian oscillations in LL (Kay and Millar, 1993; Suarez-Lopez et al., 2001). To our knowledge, there has so far been no report of P. patens showing clear circadian rhythm in LL. It is an interesting question whether this arrhythmicity in LL is specific only to particular ccgs or is a fundamental property of the circadian clock itself in P. patens. This question is important especially from the evolutionary point of view, because if the latter is the case, it is indicated that regulatory mechanisms of the circadian clocks are significantly diverged between P. patens and higher plants. This issue will be clarified by cloning and expression analysis of clock genes in P. patens.

The three PpSig genes were all directly induced by light (Fig. 7). However, the profiles of the PpSig1 and PpSig2 expression in LD showed very little, if any, light responsiveness at the transition from dark to light (Fig. 2B). PpSig5 showed a clear peak in the light phase; however, judged from kinetics, this peak is unlikely to contain a component of the direct response to light, but is likely to be exclusively a circadian peak (Fig. 2B). Therefore, direct response to light cannot be clearly seen in any of the PpSig genes in LD. Because the responses to light shown in Figure 7 were induced after cells were kept in the dark for 24 h, the ability to respond to light may be only observed for cells in such dark-adapted cells. Alternatively, the response to light of the sigma genes may be circadian clock-dependent as reported for Lhcb genes in higher plants (Millar and Kay, 1996; Sugiyama et al., 2001), in which the amplitude and kinetics of the acute response changed depending on the circadian time at which a light pulse is administered.

The blue light signal, at least partly mediated by the cryptochrome(s) PpCRY1a and/or PpCRY1b, induced PpSig1 and PpRpoA genes (Fig. 7). In addition, the results also indicated the involvement of a blue light photoreceptor(s) other than the two cryptochromes in light induction of PpSig1 and PpSig5 (Fig. 7).

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Light Sources

Physcomitrella patens subsp. patens (Ashton and Cove, 1977) was maintained in LD using white fluorescent lamps as the light source (light intensity approximately 40 μmol m⁻² s⁻¹) at 25°C. Protonema cells were grown on BCD medium supplemented with 1 mM CaCl₂ and 5 mM ammonium tartrate (Nishiyama et al., 2000); the cells were collected every 7 d, and were ground with a homogenizer (Physocotron, Microtec Nition, Chiba, Japan) before they were applied to a new BCD medium supplemented with 1 mM CaCl₂ and 5 mM ammonium tartrate agar plate. The light-emitting diodes (LEDs) used as blue light sources in the light induction and blue light-dark cycle experiments was STICK LED (λmax = 470 nm at 40 μmol m⁻² s⁻¹; Tokyo Rikakikai, Tokyo).

Molecular Cloning of PpSig5 cDNA and Gene from P. patens

We amplified a DNA fragment corresponding to an expressed sequence tag (accession no. BI894524) from P. patens cDNA by PCR using two primers: Sig5probeU2 (5'-GGGAAAATACAGCCCATGAATGA-3') and Sig5probeL1 (5'-CCTCATCATTGCGAGGACATGAC-3'). The PCR product with the expected size of 461 bp was used as a probe to screen a P. patens cDNA library (gift from the Physcomitrella EST Programme; 1 × 10¹⁰ pfu/2°C. Protonema cells were grown on BCD medium supplemented with 1 mM CaCl₂ and 5 mM ammonium tartrate agar plate. The light-emitting diodes (LEDs) used as blue light sources in the light induction and blue light-dark cycle experiments was STICK LED (λmax = 470 nm at 40 μmol m⁻² s⁻¹; Tokyo Rikakikai, Tokyo).

Semiquantitative RT-PCR Analysis

Semiquantitative RT-PCR analysis was conducted as previously described (Aoki et al., 2004) with minor modifications. Protonema cells that had been cultured for more than 3 weeks in LD were harvested and frozen at intervals indicated in each figure. Total RNA was extracted from frozen cells with a kit (RNAeasy Plant Mini kit; Qiagen, Valencia, CA). The extracted RNA (1 μg) was reverse-transcribed by using M-MLV reverse transcriptase (Invitrogen) with oligo(dT)₁₂₋₁₈ primer (total volume of the reaction mixture was 25 μL), and 1/173 of the reaction mixture was subjected to semiquantitative PCR.
analysis. The primers for PCR analysis were as follows: for PsSig1 hara2isita (5'-AAATCCGCGAGTCGTTCTGCAGG-3'); and ESTprimer (5'-ACTGTCAGAGTGGCTGCTGCGAAGAGAGAC-3'). For PsSig2 PR991119/1 (5'-GGTGAATGGATATCTGGAGGCT-3') and PR900085/1 (5'-AGGCTTATCAGACGGTCTTGT-3'). For PsSig5 Sig5SLS (5'-GAGGCGACCTGACGATGAAAGC-3') and PW-U3forSig5 (5'-GTGGCCTGTTCATTTGACTTCTC-3'). For PsRpA RpoA-1 (5'-GGTGAAGCTTGGAGACGTCTG-3') and RpoA-2 (5'-GGATGGCTGTCGCTTCTGCTAAGT-3'). For PsActin1 U1 for Actin 1 (5'-GTGGCCTGAGCATACCCCATTC-3').

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LITERATURE CITED


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